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Do complement factor H 402Y and C7 M allotypes predispose to (typical) haemolytic uraemic syndrome?

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Summary

Typical haemolytic uraemic syndrome (HUS) is mainly caused by infections with enterohaemorrhagic *Escherichia coli*, whereas in atypical, nonbacteria-associated HUS, complement plays a dominant role. Recently, complement has also been shown to be involved in typical HUS. In this study, mostly weakly significant associations with homozygosities of complement allotype C7 M and inversely with factor H 402H were found, suggesting that 402Y and C7 M allotypes predispose to (typical) haemolytic uraemic syndrome.

Typical haemolytic uraemic syndrome (HUS), a severe renal disease, is mainly caused by infections with enterohaemorrhagic *Escherichia coli* (EHEC) strains. EHEC produce several virulence factors, of which Shiga toxins are believed to play a dominant role (Orth *et al.*, 2007). About 10% of all HUS cases are not caused by bacteria and are thus termed atypical HUS. Many of these are dependent on complement malfunctions. These are observed as familial or sporadic forms and recurrences are possible (Rodriguez de Cordoba & Goicoechea de Jorge, 2008). The most common cause for atypical HUS is dysregulation of the alternative pathway of the complement system.

Mutations have been described for complement factor H (FH; Warwicker *et al.*, 1998; Perez-Caballero *et al.*, 2001), a central regulator of the alternative pathway (Speth *et al.*, 2008), which is a soluble protein with a molecular weight of 150 kDa and consists of 20 short consensus repeats (SCRs) or complement control protein repeats (CCPs; Atkinson & Goodship, 2007). SCRs 19–20 are a hot spot for mutations associated with HUS (Caprioli *et al.*, 2001; Richards *et al.*, 2001; Saunders *et al.*, 2007). Development of antibodies against FH, predominantly in adolescent patients, may represent a further complication in atypical HUS (Dragon-Durey *et al.*, 2005; Jozsi *et al.*, 2007; Skerka *et al.*, 2009). It has been shown, that antibodies against FH predominantly bind to SCRs 19–20 (Jozsi *et al.*, 2007).

Atypical HUS is not only associated with mutations in regulator genes of complement but also with allotypes of the same proteins (Caprioli *et al.*, 2003; Sullivan *et al.*, 2010). Many studies have been performed in recent years to investigate a polymorphism at codon 402 (position 384 in mature complement FH), which was first described in 1988 (Day *et al.*, 1988). This polymorphism is located in SCRs 6–8 and characterized by a substitution of tyrosine to histidine. A significant correlation was found between allotype 402H of this FH polymorphism and age-related macular degeneration (AMD); a more than sevenfold higher risk was found (Edwards *et al.*, 2005; Hageman *et al.*, 2005; Haines *et al.*, 2005; Klein *et al.*, 2005). AMD is the main cause of blindness or partial sight impairment, respectively, in the older population (Evans *et al.*, 2004; Friedman *et al.*, 2004).

Thirty years ago, Monnens *et al.*, have already found increased breakdown products of C3 and factor B in the serum of EHEC-caused typical HUS patients, suggesting an activation of the complement system, possibly via the alternative pathway; however, no definite explanation for these observations was given (Monnens *et al.*, 1980). Recently a role of complement in typical HUS was demonstrated by the finding that Shiga toxin 2 binds to FH; this binding is in particular directed to SCRs 6–8 and SCRs 18–20 and leads to a reduced FH co-factor activity on the cell surface (Orth *et al.*, 2009). The involvement of alternative pathway activation in typical HUS was corroborated by Thurman *et al.* (2009) who showed significantly increased plasma levels of complement activation products Bb and SC5b-9 in these patients.

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Only 15% of the infected patients under 10 years of age progress to typical HUS, but apart from very young or very old age, no host predisposition factors are known (Karch *et al.*, 2005). As we have shown that complement is involved in typical HUS, the aim of our present study was to analyse whether allotypes of complement regulatory proteins are associated with typical HUS. For the reasons described above, we evaluated the association of FH Y402H alleles, but also included the alleles of the C7 M/N polymorphism. C7 is a central protein of the terminal complement cascade and an integral part of the membrane attack complex. Recent findings corroborate that C7 is also a regulator protein (Würzner, 2000; Bossi *et al.*, 2009). C7 is polymorphic, and one polymorphism is based on the reactivity of an allospecific mouse monoclonal antibody (mab) that detects two alleles (*C7*M* and *C7*N* allele) or three phenotypic features (C7 M, C7 MN and C7 N) (Würzner *et al.*, 1992). The genetic basis, assessed using epitope mapping, has been assigned to a single nucleotide substitution of codon 565 (Würzner *et al.*, 1995a) causing a marked conformation change of the protein leading to a lower secretion into the serum ('hypomorphic' appearance), the reason why it was thought to represent a good candidate, although previous studies have not shown significant disease associations with that allele (Würzner *et al.*, 1995b).

Eighty-three serum samples of typical HUS patients were tested for allotypes of the FH Y402H and the C7 M/N polymorphism; 31 sera were from the Austrian Reference Laboratory for EHEC at the Division of Hygiene and Medical Microbiology, Innsbruck Medical University, Austria, and 52 sera were from the HUS Reference Centre at the Institute of Hygiene, University of Münster, Germany. IgM and IgG antibodies against O157:H7, the most common EHEC serotype worldwide (Scheiring *et al.*, 2008), have been detected for all samples. The sera were stored at −20°C. Multiple freezing and thawing was avoided.

For determination of the allotype of the FH Y402H polymorphism, an ELISA-based test kit was used (Hycult, Uden, the Netherlands). The samples were prepared according to the manufacturers' instructions, samples with known allotypes (FH 402H, FH 402YH, FH 402Y) and dilution buffer were used as controls. Measurement using ELISA reader was recorded at a wavelength of 450 nm.

Typing of C7 M/N was performed as published (Würzner *et al.*, 1990). Briefly, homozygous and heterozygous individuals are identified by the ratios of C7 concentration of two ELISAs, one based on the allospecific mab WU 4–15 (ELISA M), and the other based on the polyclonal goat anti-C7 IgG (ELISA P) as coating antibodies. After blocking, serial diluted-normal human serum and diluted serum from HUS patients (1:2000) were introduced to each ELISA well for 1.5 h. The plates were incubated with biotinylated goat anti-C7 IgG for 1 h followed by alkaline phosphatase-conjugated avidin (1:500) and 4-nitrophenylphosphate as detecting reagents and substrate respectively. The enzyme activity was read at dual wavelengths of 415 and 490 nm. The C7 concentrations of the serum samples were determined by comparison with a standard concentration from normal human serum. Samples with an ELISA M/ELISA P ratio of 0.75–1.25 were defined as C7 M, ratios of 0.35–0.65 were defined as C7 MN, whereas ratio of 0.02 or less than defined C7 N respectively (Würzner *et al.*, 1990, 1992). More than 99% of all samples fell into one of the three ranges and could be unequivocally determined after testing in triplicates.

A chi-squared test was performed to compare the data of typical HUS patients for FH Y402H and C7 M/N allotypes with those of healthy controls. We analysed 83 serum samples of typical HUS patients, and found 41.0% of all cases having the FH 402Y allotype, 53.0% of the specimens were detected as 402YH and 6.0% of the sera were homozygous 402H. As controls, we have tested 100 age- and sex-matched healthy persons from the same geographical regions, of which 32% were homozygous 402Y, 53% were heterozygous and 15% were homozygous 402H (Fig. 1). Comparing the data, the homozygous allotype 402H was significantly less frequent ($P = 0.05$) in HUS patients than in controls.

Thakkinstian *et al.* (2006) compared eight different studies with a likely high proportion of Caucasian samples and a possibly quite comparable study group for FH Y402H allotypes in AMD patients and controls. The distribution of the three genotypes in 3326 healthy individuals was 40% for 402Y, 47% for the heterozygous 402YH and 13% for 402H, and thus quite comparable to our controls.

C7 M/N allotyping of the 83 sera from HUS patients investigated in this study showed 80.7% of the samples to be C7 M; C7 MN was found in 15.7%

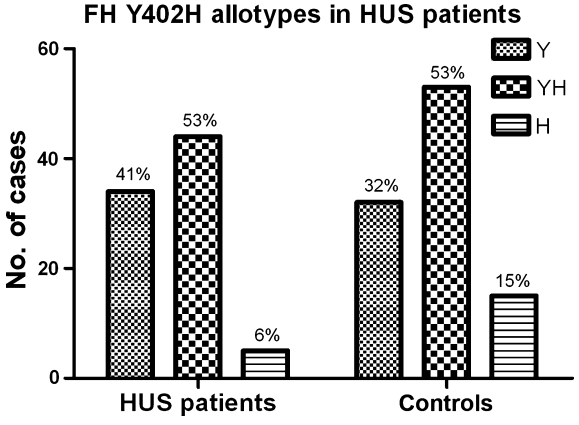

Figure 1. FH Y402H allotypes of 83 typical haemolytic uraemic syndrome (HUS) patients from Innsbruck and Münster compared with 100 healthy controls. A chi-squared test was performed and homozygous factor H 402H was found to be significantly less frequent ($P = 0.05$) in HUS patients.

samples, whereas 3.6% were C7 N. Comparing these C7 M/N allotypes with those of 100 age- and sex-matched healthy persons from the same geographical regions, 51% were C7 M, 43% heterozygous and 6% homozygous for the less frequent *C7*N* allele (Fig. 2). Comparing the data the homozygous allotype C7 M was significantly more frequent and the gene frequency of *C7*N* significantly less frequent in HUS patients than in controls (both $P < 0.01$). Gene frequencies from earlier studies in the same geographical region were comparable for healthy individuals (Würzner *et al.*, 1991).

As only 15% of all children below 10 years do get HUS after infection with EHEC, host factors are very likely to be involved. As complement has been shown to be involved not only in atypical but also in typical HUS, we tried to find out whether allotypes of two important complement proteins, FH and C7, are associated with typical HUS. FH was chosen because it is implicated in typical HUS, and the Y402H polymorphism was selected because it is located in the region where Shiga toxin binds to FH (SCRs 6–8; Orth *et al.*, 2009). C7 was chosen because it is the central modulator of the terminal pathway. Both the Y402H and the C7 M/N polymorphism were selected, because they are protein polymorphisms with two frequent alleles (Day *et al.*, 1988; Würzner *et al.*, 1992) of which the rare one of the latter, *C7*N*, is even hypomorphic (Würzner *et al.*, 1991).

Fifty-four samples from atypical HUS patients have been typed for FH Y402H, but no disease association was found (Rodriguez de Cordoba, personal communication). When a more extended haplotype was looked at, interestingly one involving FH 402Y was shown to have an odds ratio of 1.57 (Rodriguez de Cordoba & Goicoechea de Jorge, 2008).

Until now, only a small number of studies have investigated an association between infectious diseases and FH Y402H polymorphism. It was shown that

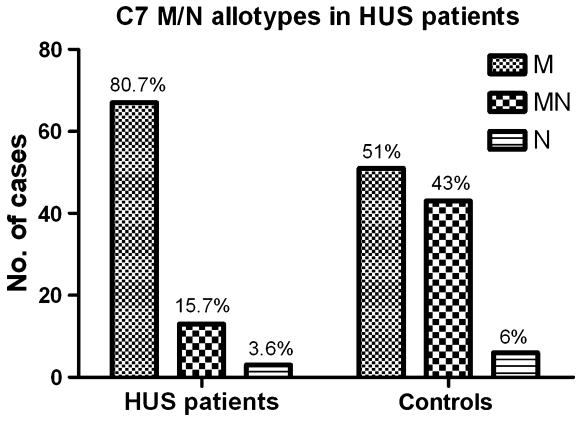

Figure 2. C7 M/N allotypes of 83 typical haemolytic uraemic syndrome (HUS) patients from Innsbruck and Münster compared with 100 healthy controls. A chi-squared test was performed and homozygous C7 M was found to be significantly more frequent and C7*N significantly less frequent (both $P < 0.01$) in HUS patients.

group A streptococcus strains had a decreased growth and an increased opsonophagocytosis in the blood of homozygous 402H individuals. Therefore, these individuals are protected against group A streptococcus infections, when they are young, but they have a high risk to develop AMD, when they become older (Haapasalo *et al.*, 2008). The significance is obviously somewhat weak, but it is possible that homozygous 402H also confers a low protection against *E. coli* causing HUS.

While determining the C7 M/N allotype, C7 concentrations have been assessed en route (Würzner *et al.*, 1990). About 97.6% had a C7 concentration within the normal range and no sample was lower than the normal range. It is therefore likely that HUS caused by EHEC has no effect in altering C7 concentrations in the serum and *vice versa*, low C7 is not predisposing to HUS. The former is interesting, as Shiga toxin 2 not only binds to FH, but also activates complement (Orth *et al.*, 2009). The activation, however, is probably concentrated locally at the kidney and not accompanied by a significant systemic decrease of C7. The quantitation of C7 in sera from patients infected with other infectious agents, such *Borrelia burgdorferi*, *Mycoplasma pneumoniae*, Rubella virus, Cytomegalo virus and Epstein–Barr virus, revealed that about 92.5% of the samples also have C7 concentrations within the normal range; among these samples which showed lower C7 levels, 83.3% were found in acute Lyme borreliosis patients (Würzner *et al.*, 1995b).

Both the homozygous C7 M allotype was significantly more frequent and the *C7*N* allele significantly less frequent in HUS patients ($P < 0.01$), whereas C7 M/N allotypes in the other infectious diseases mentioned above were not significantly different compared with healthy individuals (Würzner *et al.*, 1995b). The association of C7N with healthy subjects rather than HUS patients is in a way surprising, as the substitution of threonine by proline at codon 565 indeed results in a marked conformational change of the protein. This may also change the functions of the whole molecule as membrane anchor for the C5b-7 complex (DiScipio, 1992) and indeed causes a 'hypomorphic' appearance, i.e. a lower concentration in serum (Würzner *et al.*, 1991). Further studies have to confirm this association, but it has been speculated before that an impaired terminal complement pathway may be of advantage in severe systemic disease following a gastrointestinal infection (Lachmann, 1987).

In conclusion, in this study of FH Y402H and C7 M/N allotypes, weak associations of complement allotypes to susceptibility to typical HUS have been found. This does not exclude that other polymorphisms, which at present cannot be detected at the protein level, in particular of FH, also show associations – in any case, host factors are likely to be involved and complement and especially FH are likely candidates.

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